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F-spondin Gene Transfer Improves Memory Performance and Reduces Amyloid- β Levels in Mice

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Abstract

Alzheimer's disease (AD) is the most prevalent form of dementia affecting the elderly. Evidence has emerged signifying that stimulation of the reelin pathway should promote neural plasticity and suppresses molecular changes associated with AD, suggesting a potential therapeutic application to the disease. This was explored through the use of lentiviral vector mediated overexpression of the reelin homolog, F-spondin, which is an activator of the reelin pathway. Intrahippocampal gene transfer of F-spondin improved spatial learning/memory in the Morris Water Maze and increased exploration of the novel object in the Novel Object Recognition test in wild-type mice. F-spondin overexpression also suppressed endogenous levels of amyloid beta (A β ₄₂) in these mice and reduced A β plaque deposition and improved synaptophysin expression in transgenic mouse models of AD. These data demonstrate pathologic and cognitive improvements in mice through F-spondin overexpression.

Keywords

F-spondin; Reelin; Gene Therapy; Amyloid-beta; Alzheimer's disease

1.2 INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder currently affecting one in eight Americans over the age of 65 according to the Alzheimer's Association's Facts and Figures 2012. Much research in AD has focused on signaling proteins and their receptors in the low-density lipoprotein family. Of particular interest is the extracellular matrix protein reelin, which is a ~400 kilodalton (KD) protein whose role in development includes proper formation of cortical layers, foliation of the cerebellum, and migration and positioning of Purkinje cells (Caviness and Sidman, 1973, Caviness, 1982, D'Arcangelo et

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al., 1995, Wallace, 1999). Even in adulthood, reelin plays an important part in the positioning of newly divided neurons, synaptic plasticity and dendritic spine development (Niu et al., 2004, Heinrich et al., 2006, Gong et al., 2007, Niu et al., 2008). It has garnered attention in the field of AD due to its ability to bind to low-density lipoprotein (LDL) receptors that also bind apolipoprotein E (apoE), and polymorphisms in apoE are the best known genetic risk factor for acquiring sporadic AD (D'Arcangelo et al., 1999, Ashford, 2004). Specifically, reelin binds the very low-density lipoprotein receptor (VLDLR) and apoE receptor 2 (ApoE-R2). Upon the binding of reelin by VLDLR and ApoE-R2, a signaling cascade is initiated that is involved in regulating the activity of key kinases (reviewed in (Herz and Chen, 2006)). The expression of reelin has been reported to be reduced in AD (Chin et al., 2007) and polymorphisms in the reelin gene have been linked to the risk of developing AD (Seripa et al., 2008, Kramer et al., 2010). The role of reelin in Alzheimer's disease is quite complex due to its ability to alter multiple pathologies observed in AD. The importance of reelin likely stems from its ability to regulate kinases (GSK3, CDK5) involved in the production of the β -amyloid peptide (A β) and in phosphorylation of the microtubule-associated protein, *tau* (Hiesberger et al., 1999, Beffert et al., 2002, Ohkubo et al., 2003, Phiel et al., 2003). Additionally, reduced reelin expression has been shown to accelerate both amyloid plaque and *tau* formation in AD mouse models (Knuesel et al., 2009, Kocherhans et al., 2010). The interaction of reelin with amyloid precursor protein promotes neurite outgrowth (Hoe et al., 2009), and through its ability to modify synapses, reelin was shown to restore A β -induced reductions in LTP and improve synaptic strength in CA1 pyramidal cells (Durakoglugil et al., 2009). Taken together there is strong evidence that reelin is important to AD pathogenesis.

In addition to reelin, other ligands also bind to VLDL and ApoE-R2 including apoE, thrombospondin, and F-spondin (Strickland et al., 1995, Hoe et al., 2005a, Hoe et al., 2006). F-spondin is of particular interest as it is a homolog of reelin, possessing an N-terminal reelin domain. Like reelin, F-spondin is also a secreted extracellular protein (of smaller size, 115 kD) expressed in the hippocampus with a role in proper neuronal placement and plasticity in development and adulthood (Higashijima et al., 1997, Burstyn-Cohen et al., 1999, Feinstein et al., 1999, Andrade et al., 2007). Furthermore, F-spondin itself has been shown to regulate processing of membranous APP (Ho and Sudhof, 2004, Hoe et al., 2005b). While ample research exists for reduced reelin in AD, the effect of overstimulation is not as well defined. Moreover, F-spondin's role in AD is poorly understood despite the therapeutic potential for overexpressing ligands like F-spondin in AD. To explore this potential, a lentiviral vector was created expressing F-spondin to facilitate overexpression *in vivo*. Viral vectors were injected into the dentate gyrus of the hippocampus of both wild-type and AD-like transgenic mice. Mice were analyzed for behavioral and neurochemical alterations associated with AD.

1.3 EXPERIMENTAL PROCEDURES

1.3.1 Animals

All mice were used according to IACUC approved protocols and in accordance with the "Guide for Care and Use of Laboratory Animals", as published in 1996 by the National Academy of Sciences. AD Transgenic mice consisted of the TASD41 line (combined APP_{swe/lon} mutations) (Rockenstein et al., 2001) and the TASTPM line (carrying both mutant APP_{swe} and PS1_{M146V} transgenes) (Howlett et al., 2004, Howlett et al., 2008). Our colony of TASD41 mice have drifted genetically compared to the original colony and developed plaque pathology at a later date (~8 month). Our colony of the relatively new TASTPM line develop pathology at the expected earlier time point of ~4 months. Both models develop amyloidosis and age dependent deficits in learning and memory (Howlett et

al., 2004, Singer et al., 2005, Spencer et al., 2008) and so were good models of AD for use in our studies.

1.3.2 Lentiviral Vector Preparation

Lentiviral vectors expressing the F-spondin or GFP cDNAs were constructed and prepared as previously described (Tiscornia et al., 2006). The murine F-spondin cDNA was purchased from Open Biosystems and subcloned into pCSC-SP-PW (pBOB). The lentiviral GFP vector has been previously described (Marr et al., 2003). Vector titers were determined by flow cytometry as previously described (Marr et al., 2003).

1.3.3 Lentiviral Vector Injections

Anesthetized mice (isoflurane) were placed in a stereotaxic frame (Kopf). A single bilateral injection of two μ l of lentiviral vector solution was done with a 5 μ l Hamilton syringe (30 gauge needle) at a rate of 0.4 μ l/min using an autoinjector (Stoelting). These injections were targeted into the dentate gyrus (coordinates: 2mm caudal from bregma, $\pm$ 1.8mm lateral from midline, 2mm ventral from the dura) (1×10^6 TU/hemisphere). Wild-type mice were injected at 9 weeks of age (n=10 to 15). TASTPM littermates were injected at 5.5 months of age (n=12). TASD41 littermates were injected at 9 months of age (n=8). Equal proportions of males and females were used between the control and F-spondin groups. Note: the different rates of development of AD-like pathology in these transgenic lines dictated the different ages of lentiviral injection (*i.e.* near the time of first amyloid deposition).

1.3.4 Morris Water Maze (MWM)

Two months after lentiviral injections, mice were individually placed in a pool of water made opaque by white non-toxic water soluble paint and allowed one minute to search for a submerged platform. Each mouse underwent four 60-second trials per day for 5–7 consecutive days in the acquisition phase. Mice that did not successfully find the platform in the allotted time were placed on the platform for 30 seconds before the next trial. The movements of the mice were recorded by a video tracking system (TSE Systems, Inc., Midland, MI). At 24 and 72 hours after the last acquisition trial, the platform was removed and mice were allowed a single 1-minute swim (probe trials). The amount of time spent in the quadrant that contained the platform was determined.

1.3.5 Novel Object Recognition Task (NORT)

Following MWM, episodic-like memory was tested using the NORT. One day prior to testing, mice were acclimated to the open field box by being individually placed in the box with no objects and allowed to explore for 30 minutes. The following day, each mouse was placed in the box with 2 identical objects and allowed 10 minutes to explore. Three hours later, one object was replaced with a novel object and the mouse was placed back into the box for another 10 minutes of exploration. The time spent investigating each object and the number of visits to each object were recorded using a video tracking system (TSE Systems Inc.).

1.3.6 A β ELISA

Following transcardial perfusions with saline, brains were extracted and divided into left and right hemispheres. For analysis of A β , one hippocampus was hemisected and snap frozen. Frozen brain sections were homogenized in lysis buffer (5M Guanidine-HCl, Fisher Scientific) using a Polytron homogenizer (Hafez et al., 2011). After centrifugation at 16,000xg (30 min), supernatants were collected and diluted (1:10) for analysis of A β . Quantification of A β ₁₋₄₂ was performed using an isoform specific A β ELISA kit (Wako

Chemicals) (Iwata et al., 2001, Hafez et al., 2011). Protein levels were quantified using a BCA quantification kit (Pierce) to normalize measured A β levels.

1.3.7 Immunohistology (IH) Analysis

IH analyses were performed using the remaining hemisected brain (see A β ELISA). Brains were immersion-fixed in a 4% paraformaldehyde fixative solution for 48 hours before being placed in a cryoprotectant solution (30%-sucrose, 0.1M PO₄). Fixed hemi-brains were sagittally sectioned on a freezing microtome (40 μ m) and stored in a cryoprotectant solution at -20°C. F-spondin expression was detected using the SPON1 antibody (1:250, Abcam). A plaque load was detected using the antibody 4G8 (1:250, Sigma). Prior to application of 4G8 antibody, brain tissue underwent pretreatment with 70% formic acid for 10 minutes for antigen recovery. Synaptophysin staining was elucidated using the Syn-38 antibody (1:500, Abcam). After application of the primary antibodies, samples were incubated for 48 hours at 4°C prior to applying secondary antibody (2 hours, Alexafluor 594 goat anti-rabbit 1:250, or Alexafluor-594 goat anti-mouse 1:250, Invitrogen) and imaged by fluorescent or laser scanning confocal microscopy. MetaMorph® software was used for densitometric analyses. Densitometric analysis of A β plaque burden was quantified by averaging the percent area staining positive (above threshold) within the entire hippocampal region from several sections per animal. Densitometric analysis of synaptophysin staining was accomplished by randomized sampling throughout the region of the dentate gyrus of the hippocampus at high magnification (60X).

1.3.8 Statistics

Data analysis was performed using the statistical software JMP 7.0.1. For the water maze experiments, a repeated-measures ANOVA was used with the grouping factor being the treated animals (Lenti-F-spondin or GFP), and repeated measures being the acquisition phase trial days. Water maze probe trials (time in correct quadrant and platform crossings) as well as novel object data (exploration time, recognition index, visits) were analyzed using one-way ANOVA. Means for A β ELISA and densitometric analyses were also compared using one-way ANOVA. Tukey-Kramer HSD post hoc paired comparison tests were run on all significant effects with $p < 0.05$.

1.4 RESULTS

1.4.1 F-spondin overexpression improves memory performance in wild-type mice

A lentiviral vector expressing murine F-spondin under the control of the human cytomegalovirus (CMV) promoter was constructed. Expression was confirmed by Western blot for transduced cells *in vitro* (not shown). Wild-type mice were injected into the dentate gyrus (dg) with a lentiviral gene transfer vector expressing mouse F-spondin or GFP control. Two months post injections mice were tested for learning and memory proficiency in the MWM and NORT. Following behavior testing, mice were sacrificed by transcardial saline perfusion and the hemibrains collected for analyses. Fixed and sectioned hemibrains showed clear expression of F-spondin in the dg by immunohistology (IH) after lentiviral F-spondin gene transfer compared to lentiviral GFP control (Fig. 1). The increased F-spondin signal was not seen outside the hippocampus and was concentrated in the dentate gyrus. While the increased F-spondin signal was clear after gene transfer is difficult to determine how much more is produced over endogenous levels.

The MWM is designed to test the acquisition and retention of spatial memory. F-spondin-injected mice demonstrated an improved acquisition of the platform location compared to GFP mice (Figure 2A). In the probe trials (B, C), F-spondin mice spent a significant amount of more time in the platform quadrant compared to GFP treated mice at 72 hrs (Figure 2C).

No changes in swim speed were found (data not shown). NORT was done to assess episodic-like memory in treated mice. F-spondin gene transfer significantly increased the time exploring the new object compared to the old object relative to GFP (Figure 3A). Additionally, F-spondin mice spent significantly more time exploring both objects (Figure 3B), indicating enhanced exploratory behavior and perhaps reduced anxiety. Despite the improved memory performance, the levels of synaptophysin staining were not different between GFP and F-spondin groups as measured by IH (data not shown).

1.4.2 F-spondin overexpression reduced A β levels in wild-type mice

Production of A β is believed to be a key component of AD pathology. Recent evidence suggests that lowered stimulation of the reelin pathway exacerbates AD-like pathology in APP-transgenic mice, including the induction of A β accumulation (Kocherhans et al., 2010). To determine the effect of reelin-pathway stimulation on endogenous A β levels, injected hippocampi were dissected and total A β was extracted for quantification by ELISA. F-spondin mice showed a significant reduction in A β_{42} compared to GFP treated mice (Fig. 4).

1.4.3 F-spondin overexpression reduced amyloid-beta pathology in AD-transgenic mice

Transgenic mice over-expressing human mutant APP and PS1 are a standard model for AD that develop many AD-like pathological alterations (Rockenstein et al., 2001, Howlett et al., 2004). To test the therapeutic potential of F-spondin gene transfer, we injected AD transgenic mice with lentiviral F-spondin and GFP vectors (as above). When measuring A β plaque burden by IH, F-spondin treated transgenic mice possessed significantly fewer plaques in the hippocampus compared to GFP mice (Fig. 5) (Cerebral cortex was not affected, data not shown). However, no effect was found on total extractable A β_{42} by ELISA (data not shown). Analysis of synaptic density through synaptophysin IH showed a significant increase in the neuropil expressing synaptophysin in APP transgenic mice treated with F-spondin (Fig. 6).

Evidence supports a role for reelin signaling in promoting synaptic strength (Beffert et al., 2002, Assadi et al., 2003, Niu et al., 2004, Niu et al., 2008). However, the reduced plaque burden and increased synaptophysin did not have a measurable effect on memory. There were no improvements in performance produced by F-spondin in the TASTPM AD-transgenic mice in the MWM (GFP n=7, F-spondin n=5, data not shown) suggesting that the therapeutic effects of F-spondin were not sufficient to improve spatial memory in this aggressive model of amyloidosis. Examination of these mice in the NORT was inconclusive. While F-spondin treated mice spent significantly more time at the new object compared to the GFP group, both F-spondin and GFP groups spent more time on the old object compared to the new object which is unusual rodent behavior (Dodart et al., 2002). However, when examining the number of visits to the new object compared to total visits to objects (new + old), F-spondin mice visited the novel object significantly more than GFP treated mice at about 60% of the time ($p<0.05$) (data not shown).

1.5 DISCUSSION

Our results showing improved memory with F-spondin treatment are consistent with previous reports that stimulation and suppression of the reelin pathway improve and impair memory, respectively (Brosda et al., 2011, Rogers et al., 2011). This beneficial effect on memory may be linked to the promotion of synaptic plasticity by F-spondin. We also observed behavioral changes that are consistent with reduced anxiety. Similar effects of reelin stimulation on depression-like behavior have been reported for transgenic mice overexpressing reelin (Teixeira et al., 2011).

Treatment with F-spondin was also shown to suppress endogenous production of A β ₄₂. This is notable as these mice lack any artificial human mutant familial-AD-associated transgenes and do not overproduce A β . Therefore, these mice are a better indicator of the *in vivo* biology of sporadic AD (comprising the vast majority of cases). These data suggest that the overproduction of F-spondin could be therapeutic in AD through the suppression of A β production. However, we do not believe that the beneficial effects on memory (Figs. 2 and 3) are necessarily due to the reduction in A β as these endogenous levels are far lower than those produced in transgenic mouse models of AD which develop spatial and episodic-like memory deficits.

Amyloid plaque deposition in two APP transgenic lines was also suppressed by F-spondin, further supporting a potential therapeutic application of F-spondin to AD. However, biochemical analysis of total A β ₄₂ did not show a significant effect by F-spondin. This is somewhat paradoxical as plaque load measured by IH showed a clear reduction. It is possible that the extraction buffer used for A β quantitation by ELISA dissolved the large amount of both diffuse and fibrillar forms of A β , occluding the detection of any reductions in soluble A β measured on a molar level. We also did not find a substantial effect on learning and memory in the APP transgenic mice. It is notable that there was a clear upregulation of synaptophysin expression induced by F-spondin gene transfer (Fig. 6). This could be due to the trophic effects of this ligand on synaptic plasticity or a result of lowered plaque deposition (or perhaps both). However, this was not associated with a clear improvement in memory in APP transgenic animals. It should be noted that even the increased levels of synaptophysin produced by F-spondin in the APP transgenic line used in the memory tests (12.5 $\pm$ 4.9% of surface area above threshold) did not reach levels seen in non-transgenic mice (42.8 $\pm$ 11.8% of surface area above threshold). Perhaps, greater effects on A β load and memory could have been facilitated with the injection of more viral vector or longer durations of expression. Recently, it has been shown that reelin expression is correlated with post-synaptic density – 95 (PSD-95) protein (Ventruti et al., 2011). It will be interesting to determine the effects of F-spondin gene transfer on this synaptic marker as well. Studies have found that F-spondin levels in the cerebrospinal fluid (CSF) are increased in association with AD. Ringman and colleagues found a 19% increase in F-spondin protein in the CSF when comparing the symptomatic/presymptomatic FAD mutation carriers with related non-FAD mutation carriers (Ringman et al., 2012b). Similarly, Zhang and colleagues found a substantial elevation in CSF F-spondin in one cohort of AD patients compared to age matched non-demented controls (Zhang et al., 2005). While this supports our study in linking F-spondin to AD the increase of expression associated with AD suggests that elevated F-spondin may be part of the disease pathogenesis or at least not protective from AD. It should be noted that these are measurements of CSF protein which do not necessarily correlate directly with expression levels in the brain. A common example of this is the finding that A β ₄₂ levels are reduced in the CSF in association with AD (Ringman et al., 2012a) while it is known that it is increased in the brain. Furthermore, elevated F-spondin could be a homeostatic response to reduced reelin expression in the brain during AD.

In summary, this work is significant because it is the first study to examine the effect of constitutive reelin pathway ligand overproduction on AD-linked pathology in wild-type and Alzheimer's disease mouse models. Additionally, it is the only study to specifically assess F-spondin's role in modulating AD-like pathology *in vivo*. Data from these experiments demonstrate lentiviral F-spondin can improve memory and reduce A β ₄₂ levels in wild-type mice as well as upregulate synaptophysin and reduce plaque deposition in AD transgenic mice. While the overexpression of reelin itself was prohibited in a lentiviral context due to the large size of the reelin cDNA (10.6kb), future experiments utilizing large capacity viral vectors (like HSV or Helper-free adenoviral vectors) could allow for the direct testing of reelin in this manner. This viral vector delivery approach for F-spondin also has implications

for the treatment of other diseases and disorders of the brain including neural repair/regeneration and schizophrenia (Brosda et al., 2011, Courtes et al., 2011, Teixeira et al., 2011).

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Highlights

- F-spondin gene transfer improves memory and lowers A β in wild type mice.
- F-spondin upregulates synaptophysin and reduces A β plaques in AD-transgenic mice.
- First direct data of the effects of F-spondin elevation on neuropathology/behavior.
- Relevant for the development of therapies for Alzheimer's disease.

Figure 1. *In vivo* detection of lentiviral vector-expressed F-spondin

Representative fluorescent images of sagittally sectioned brains injected with Lenti-GFP (A, B) or Lenti-F-spondin (C, D) (1×10^6 TU per hemisphere) into the dentate gyrus of the hippocampus, immunostained with the α -spon1 antibody against F-spondin (red). Scale bar = 100 μ M

Figure 2. Lentiviral delivery of F-spondin improved performance in the Morris Water Maze Wild-type Swiss Webster mice were injected at 9 weeks of age with a lentiviral vector expressing GFP or F-spondin. 2 months after injections, spatial memory was tested in the Morris water maze. The acquisition phase shows the average latency to platform in seconds for GFP and F-spondin lenti-injected mice (A). A Probe trial was done at 24 (B) and 72 (C) hours after the last acquisition trial (1 trail, 60 seconds). Time in the quadrant that contained the platform was recorded. Data are means $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.01$. $n = 10$ per group (Swiss Webster).

Figure 3. Lentiviral delivery of F-spondin improved performance in the Novel Object Recognition Task

2 months after lentiviral vector injections (see Fig. 2), episodic-like memory was tested using the NORT. The time spent exploring the old and new object for GFP and F-spondin treated mice was determined (A). Also the total time exploring both objects (new + old) was determined (B). Data are means $\pm$ S.E.M. * $p < 0.05$. $n = 10$ per group (Swiss Webster).

Figure 4. Lentiviral delivery of F-spondin suppressed endogenous Aβ levels

A) Hippocampal levels of total Aβ₄₂ from wild-type mice injected with lentiviral GFP and F-spondin vectors were determined by specific ELISA. Data are means $\pm$ S.E.M. ** $p < 0.01$. $n = 15$ per group (strains: 5 x line 129, 10 x Swiss Webster).

Figure 5. Lentiviral delivery of F-spondin reduced plaque levels in AD transgenic mice
 Representative confocal images showing immunostained A β plaques (red) from a mouse (TASTPM) injected with lentiviral GFP (A) and F-spondin (B) (Scale bar = 200 μ m). Quantitation of A β plaque burden in AD transgenic mice injected with lenti-GFP and F-spondin (C). Data are presented as the percentage of surface area staining positive for A β relative to GFP control treated mice. Data are means $\pm$ S.E.M. * $p < 0.05$. $n = 12$ for GFP (strains: 5 x TASD41, 7 x TASTPM). $n = 8$ for F-spondin (strains: 3 x TASD41, 5 x TASTPM).

Figure 6. Lentiviral delivery of F-spondin increased synaptophysin immunoreactivity in AD transgenic mice

Representative confocal images of synaptophysin immunoreactivity are shown for GFP (A) and F-spondin (B) treated mice (Scale bar = 100 μ m). Quantitation of the synaptophysin positive surface area (above threshold) is shown (C). Data are reported as average staining by percent area relative to lenti-GFP injected mice. Data are means $\pm$ S.E.M. ** $p=0.01$. $n = 12$ for GFP (strains: 5 x TASD41, 7 x TASTPM). $n = 8$ for F-spondin (strains: 3 x TASD41, 5 x TASTPM).